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(54) Title: PROCESS FOR THE SELECTIVE ENZYMATIC HYDROLYSIS OF NUCLEOSIDE POLYESTERS

(57) Abstract: There is described a process for the selective deprotection of hydroxy groups of nucleosides polyesters by selective hydrolysis of said polyesters in an aqueous solvent in the presence of a lipase of animal or microbial origin immobilized on a solid hydrophobic support.

PROCESS FOR THE SELECTIVE ENZYMATIC HYDROLYSIS OF NUCLEOSIDE POLYESTERS

OBJECT OF THE INVENTION

The present invention refers to a process for the selective deprotection of hydroxyl functions of polyesters, in particular of di- or tri-esters, of nucleosides. More particularly, the invention refers to the selective hydrolysis in the positions 3' and/or 5' of 2', 3',5'-tri-O-acylribonucleosides or in the position 3' or 5' of 3',5'-di-O-acyl-2'-deoxyribonucleosides in the presence of a lipase immobilized on a hydrophobic support and in an aqueous solvent to obtain corresponding 2',5'(or 2',3')-di-O-acylribonucleosides, or 2'-O-acylribonucleosides or 5'(or 3')-O-acyl-2'-deoxyribonucleosides respectively.

DESCRIPTION OF THE PRIOR ART

The selective deprotection of 2',3',5'-tri-O-acetylnucleosides, catalyzed by a lipase of microbial or animal origin is described in the literature.

In particular, literature discloses the selective removal of the 5'-O-acetyl group from the 2',3',5'-tri-O-acetyluridine and of its 2'-C-methyl derivative from the 2',3',5'-tri-O-acetyl-inosine and 3',5'-di-O-acetyl-2'-deoxyinosine by hydrolysis in the presence of a porcine pancreas lipase in a buffer (H. K. Singh et al., Tetrahedron Letter, 1993, 34, 5201-5204) or, respectively, of a *Candida antarctica* in ethanol (L. E. Iglesias et al., Biotechnology Letters, 2000, 22, 361-365) or in a buffer (P. Ciufedda et al. Bioorg. Chem. Med. Lett., 1999, 9, 1577-1582).

The selective removal of the 3-hexanoyl group of the 3',5'-di-O-hexanoyl-2'-deoxyuridine and of derivatives thereof by hydrolysis in the presence of a *Pseudomonas fluorescens* lipase was also described by A. Uemura et al. in Tetrahedron Letters, 1989, 30, 3819-3820.

SUMMARY OF THE INVENTION

The above-mentioned documents state that the methods disclosed therein selectively afford the 2',3'-di-O-acetyl-derivatives or the 3'-mono-O-acetyl-2'-deoxy-derivatives or the 5'-mono-O-hexanoyl-2'-deoxy-derivatives but, actually, the selectivity of the hydrolysis is relative because, for example, the hydrolysis described by A. Uemura et al. as cited above meanly affords the 5'-mono-hexanoyl-2'-deoxy-derivative, but also affords a given amount of the completely deprotected derivative. Furthermore, the same deacylation method is not adapted for an industrial scale-up.

In addition, these methods cannot be considered of general use for the lipases of microbial origin. In fact, for example, the lipase from *Candida rugosa* is indicated

as inactive in the publication by D. I. Roncaglia et al., Biotechnology Letters, 2001, 23, 1439-1443, as it results from the fact that, in the enzymatic hydrolysis of 2',3',5'-tri-O-acetylpurines in a buffer or in mixtures buffer/dioxane or buffer/acetonitrile, it leads to the formation of totally deacetylated products.

Furthermore the patent US 5,712,099 confirm the use of lipases, such as lipase from porcine pancreas or from *Mucor* sp., even when immobilized on a support such as Eupergit®, for the preparation of arabinonucleosides totally deacetylated from corresponding triacetates.

It has now been found that the immobilization of said lipases on a hydrophobic support allows the preparation of enzymatic catalysts endowed with high activity and regioselectivity as well as with high stability. Such catalysts allow the hydrolysis of only one or both of the 3' and 5' acyl groups of 2',3',5'-tri-O-acylribonucleosides or of only one of the 3' and 5' acyl groups of 3',5'-di-O-acyl-2'-deoxyribonuclosides.

In particular, it has been found that the hydrolysis of 2',3',5'-tri-O-acetyl-uridine with a lipase of microbial origin, in particular deriving either from strains of the *Candida* genus or from strains of the *Pseudomonas* genus, respectively, leads to a selective O-deacylation in 5' or, respectively, in 3' if said lipase is immobilized on a hydrophobic support and if said hydrolysis is carried out in an aqueous solvent at a pH of from 5 to 9. Thus, contrary to the literature indications, by hydrolyzing under these conditions for example the 2',3',5'-tri-O-acetyluridine, only the 2',3'-di-O-acetyluridine with lipase from *Candida rugosa* or only the 2'-5'-di-O-acetyluridine with lipase from *Pseudomonas fluorescens* are obtained in good yields. The products obtained are easily isolated using conventional techniques.

It has also been found that 2',3',5'-tri-O-acetyl-5-fluorouridine undergoes a selective 5'-O-deacetylation if the hydrolysis is carried out with a lipase from *Pseudomonas fluorescens, Pseudomonas cepacia or Candida rugosa* immobilized on a hydrophobic support.

Moreover, it has been found that the hydrolysis of a 3',5'-di-O-acetyl-2'-deoxy-5-methyluridine (3',5'-di-O-acetylthymidine) with a lipase of animal origin, specifically with the lipase from porcine pancreas, immobilized on a hydrophobic support, selectively affords the corresponding 5'-O-acetylthymidine which can be isolated by conventional techniques.

Finally, it has been found that, by hydrolyzing the N-*n*-butanoyl-2',3',5'-tri-O-*n*-butanoyladenosine with a lipase from *Candida rugosa* or with a lipase from

Pseudomonas fluorescens immobilized on a hydrophobic support, a selective 5'-O-deacylation occurs. By a further hydrolysis of the N-*n*-butanoyl-2',3'-di-O-*n*-butanoyladenosine in the presence respectively of a lipase from *Candida rugosa* or from *Pseudomonas fluorescens* immobilized on a hydrophobic support a selective 3'-O-deacylation occurs, the 2'-*n*-butanoyloxy group remaining unaltered. By using lipase from *Candida rugosa* or from *Pseudomonas fluorescens* in suitable experimental conditions, such as a longer reaction time, a selective "*one pot*" 3',5'-di-O-deacylation occurs, the 2'-*n*-butanoyloxy group remaining unaltered. Furthermore this "*one pot*" 3',5'-di-O-deacylation also may generally occur with other lipases, such as with lipases from porcine pancreas, from *Pseudomonas cepacia* or *Rhizomucor miehei*, immobilized on a hydrophobic support.

DETAILED DESCRIPTION OF THE INVENTION

Thus, it is an object of the present invention to provide a process for the selective deacylation in position 5' or 3', or in both of them, of a 2',3',5'-tri-O-acylribonucleoside or in position 5' or 3' of a 3',5'-di-O-acyl-2'-deoxyribonucleoside, which comprises treating said 2',3',5'-tri-O-acylribonucleoside or 3',5'-di-O-acyl-2'-deoxyribonucleoside with a lipase immobilized on a hydrophobic support in an aqueous solvent at a pH of from 5 to 9.

The expression "2',3',5'-tri-O-acylribonucleoside or 3',5'-di-O-acyl-2'-deoxyribonucleoside", in the context of the present invention, includes 2',3',5'-tri-O-acylribonucleosides and 3',5'-di-O-acyl-2'-deoxyribonucleosides variously substituted on the radical of the base, hereinafter designated "nucleoside base", which can be a triazole, imidazole or purine base, and/or on the sugar radical.

The expression "aqueous solvent", as used herein, designates a solvent formed by water, for the most part, and by a water-miscible organic solvent.

Advantageously, the aqueous solvent in which the hydrolysis is performed consists of water and of a water-miscible solvent selected from the group consisting of polar aprotic solvents such as dimethyl formamide, dimethyl acetamide, dimethyl sulfoxide or, preferably, acetonitrile, wherein water represents at least 60%, practically 60-90%, more advantageously 70-80% and the water-miscible solvent represents 40-10%, more advantageously 30-20%.

The pH, which may vary from 5 to 9, advantageously from 6.5 to 7.5 and will preferably about 7, is maintained by a buffer such as a buffer formed by alkaline metal phosphates, advantageously a KH_2PO_4 buffer in a concentration which may

vary from 10 and 100 mM and will preferably be of about 25mM, hereinbelow simply referred to as "phosphate buffer".

According to a preferred embodiment, the selective hydrolysis is carried out in phosphate buffer/acetonitrile in a ratio of from 70/30 to 80/20 and at a pH of from 6.5 to 7.5, preferably of about 7, by using a lipase suitably immobilized on a hydrophobic support

The lipase used as a catalyst for the selective hydrolysis may be anyone of the lipases coming from animal or microbial sources, such as a lipase obtainable from porcine pancreas or from a microorganism, for example from *Humicola, Aspergillus, Rhizopus such as Rhizopus arrhizus, Mucor, Rhizomucor* such as *Rhizomucor miehei, Candida* or *Pseudomonas.* Advantageous lipase from animal origin is that from porcine pancreas and advantageous microbial lipases are those obtainable from microorganisms of the genus *Candida* and of the genus *Pseudomonas.*

The lipase from Candida may be obtained from *Candida rugosa, Candida antarctica, Candida lipolytica, Candida utilis,* that from *Candida rugosa* being preferred.

The lipase from *Pseudomonas* may be obtained from *Pseudomonas putida, Pseudomonas pseudoalkaligenes, Pseudomonas alcaligenes, Pseudomonas cepacia* or, advantageously, from *Pseudomonas fluorescens.*

Preferably, said lipase is selected from the group consisting of those obtainable from *Candida rugusa* or from a microorganism expressing the coding sequence which is present in *Candida rugosa* or is cloned from it (herein referred to as "Candida Rugosa Lipase"), from *Pseudomonas fluorescens* or from a microorganism expressing the coding sequence which is present in *Pseudomonas fluorescens* or is cloned from it (herein referred to as "Pseudomonas Fluorescens Lipase") and from porcine pancreas or from an animal organism expressing the same coding sequence (or a close homologue thereof) or is cloned from it (herein referred to as "Porcine Pancreatic Lipase"), said lipases being immobilized on a hydrophobic support.

The expression "hydrophobic support" designates a matrix containing hydrophobic chemical groups, such as for example alkyl chains or other hydrophobic residues as well as a matrix containing a number of suitably modified, not hydrophobic groups such as, for example, epoxy groups which, appropriately derivatized with reacting groups containing hydrophobic moieties, confer a higher degree of hydrophobicity to the support.

The expression "degree of hydrophobicity" means the percent of hydrophobic groups which are present on the surface of the support. As a consequence, two resins having "equal degree of hydrophobicity" will present, on their surface, identical hydrophobic residues or residues endowed with comparable hydrophobic characteristics.

The immobilization of the lipase is normally made on a solid hydrophobic support, such as, for example on a silicon matrix consisting of an organosilycic compound, namely of a compound containing at least a Si-C bond (US 6,080,402), on a macroporous matrix of silica or silicates (EP 444092), on a matrix consisting of adsorbing, optionally reticulated acrylic resins such as Amberlite® XAD-8 or Lewatit® E 2001/85 (EP 529424), of an amphiphilic support containing lipophilic chains (US 5,182,201), on a styrene and divinylbenzene matrix optionally containing epoxy groups such as Lewatit® R259 K or R 260 K or Diaion® HP-40, on a polyacrylic resin containing epoxy groups such as FP 4000, on a polymethacrylic resin containing epoxy groups such as Sepabeads® FP-EP or Eupergit® C appropriately derivatized with hydrophobic groups. Advantageously, the immobilization may be made on a octyl agarose gel such as Octyl Sepharose® CL-4B or on polymetacrylate based resin having a butyl character such as Sepabeads® FP-BU or a octyl character such as Sepabeads® FP-RPOD which are already totally derivatized with hydrophobic groups, said hydrophobic groups being butyl or decaoctyl chains, respectively.

Preferred selective hydrolysis catalysts are the Candida Rugosa Lipase, the Pseudomonas Fluorescens Lipase and the Porcine Pancreas Lipase, said lipases being immobilized on an octyl agarose gel, in particular on Octyl Sepharose® CL-4B or on resins presenting comparable hydrophobic groups, namely having a hydrophobicity degree equal or higher than that of said octyl-agarose gel.

The immobilization on an octyl agarose gel, or on resins wherein comparable hydrophobic groups are present or may be introduced, is normally carried out by dissolving the enzyme in phosphate buffer 10-50 mM at the selected pH, advantageously at a pH of from 6.5 to 7.5, preferably in phosphate buffer at a pH of about 7, by adding to the solution the octyl agarose gel washed with the same phosphate buffer at the same pH, by keeping the mixture under stirring at room temperature and by filtering the immobilized enzyme thus obtained. In general, the Bradford assay on the filtrate shows that only 5-20% of the enzyme is not

immobilized and that the obtained enzyme immobilized on an octyl agarose gel contains 10-100 mg of protein per g of gel or resin.

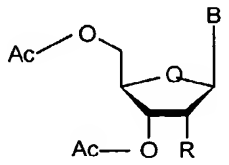
The selective hydrolysis is carried out by incubating the 2',3',5'-tri-O-acylribonucleoside or the 3',5'-di-O-acyl-2'-deoxyribonucleoside in an aqueous solvent, as defined hereinabove, at a temperature of from 4 to 40°C, normally at room temperature, for a period of time of from 30 to 75 hours in the presence of the immobilized enzyme or by passing the solution of the starting 2',3',5'-tri-O-acylribonucleoside or 3',5'-di-O-acyl-2'-deoxyribonucleoside through a column containing the immobilized enzyme.

Preferably, the aqueous solvent consists of 70-90% of 25 mM phosphate buffer at a pH of from 5 to 9, advantageously of from 6.5 to 7.5, preferably of about 7, and 30-10% of acetonitrile.

In these operative conditions the immobilized enzyme is stable and it is able to show and assume important regioselective characteristics.

At the end of the incubation, the 2'-3'-di-O-acyl-, 2',5'-di-O-acyl- or 2-O-acylribonucleoside or the 3'- or 5'-O-acyl-2'-deoxyribonucleoside is isolated according to known methods, for example by chromatography, evaporation of the solvent and crystallization of the residue or by freeze drying. The product thus isolated consists of a 3'-monodeacylribonucleoside or 5'-monodeacylribonucleoside or 3',5'-di-deacylribonucleoside in which the corresponding 2',3',5'-tri-deacyl-derivative is not detectable at the NMR spectrum at 400 MHz or of a 3'- or 5'-monodeacyl 2'-deoxyribonucleoside in which the corresponding 3',5'-di-deacyl-derivative is not detectable at the NMR spectrum at 400 MHz. If the desired product is isolated by freeze drying, it may contain a little amount of starting material which, however, does not influence the use of the desired product as an intermediate for further transformations because the starting material, totally acylated, can be easily removed in the subsequent working operations.

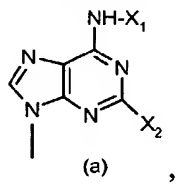
Interesting starting materials of the process of the present invention are compounds of formula II

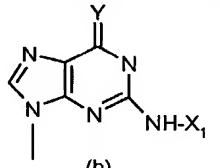


(II)

wherein B represents the radical of a nucleoside base, Ac is a (C_1-C_{18})acyl radical, R represents a hydrogen atom or a group OAc, Ac being as defined above.

Advantageous starting materials are those of formula II, wherein R and Ac are as defined above and B represents the radical of a nucleoside base selected from the group consisting of the radicals having the structures (a)-(d)


(a)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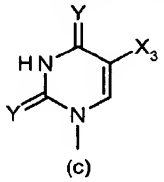
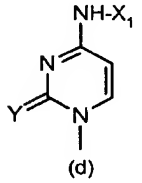

(c)

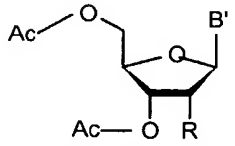

(d)

wherein X_1 represents hydrogen, a (C_1-C_4)alkyl group, an aralkyl group or a group Ac_1, Ac_1 being a (C_1-C_9)acyl group, X_2 represents hydrogen or a (C_1-C_4)alkyl group, X_3 represents hydrogen, a (C_1-C_4)alkyl group or a halogen atom and Y represents an oxygen or sulfur atom.

The alkyl group may be a saturated, linear or branched-chain aliphatic radical such as ethyl, *n*-propyl, isopropyl, *n*-butyl, or, preferably, methyl. Advantageously, the aralkyl group is benzyl, optionally substituted with a halogen atom or with a nitro or alkoxy, the ether alkyl group being as defined above.

The (C_1-C_{18})acyl radical represented by Ac may be a formyl, propanoyl, *n*-pentanoyl, *n*-hexanoyl, myristoyl, palmitoyl, stearoyl, benzoyl, phenylacetyl or, preferably, an acetyl or *n*-butanoyl group. The group Ac_1 may be a (C_1-C_9)acyl such as formyl, propanoyl, phenylpropanoyl, cyclopentylacetyl or, preferably, *n*-butanoyl, acetyl or phenylacetyl. The halogen may be chlorine, bromine, iodine or preferably fluorine. It is understood that Ac means a given (C_1-C_{18})acyl radical which is the same in the positions 3', 5' and, if any, 2'.

Interesting starting materials of the process of the present invention are compounds of formula IIa


(IIa)

wherein Ac is a (C_1-C_{18})acyl radical, R represents a hydrogen atom or a group OAc, Ac being as defined above, B' represents the radical of a nucleoside base selected from those having the formulas (b), (c) and (d) as defined above.

According to a preferred embodiment, the invention provides a process for the mono-deacylation in position 5' or 3' of a nucleoside of formula IIa above, wherein Ac is a (C_1-C_{18})acyl radical, R represents a hydrogen atom or a group OAc, Ac being as defined above, B' represents the radical of a nucleoside base selected from those having the structures (b), (c) and (d) as defined above, which comprises

treating said nucleoside with a lipase immobilized on a hydrophobic support in an aqueous solvent at a pH of from 5 to 9.

The selective hydrolysis is carried out by incubating the starting nucleoside of formula II in an aqueous solvent, as defined hereinabove, at a temperature of from 4 to 40°C, normally at room temperature, for a period of time of from 3 to 80, preferably 30 to 75 hours in the presence of the immobilized enzyme or by passing the solution of the starting nucleoside II through a column containing the immobilized enzyme.

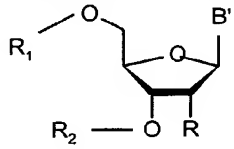
Advantageous starting materials according to this advantageous embodiment are the compounds of formula IIa, wherein R and Ac are as defined above and B' represents a radical selected from the group consisting of those having the above-defined structures (b), (c) and (d), in which X_1 is hydrogen or a (C_1-C_9)acyl, X_3 is hydrogen, fluorine or methyl and Y is oxygen. More advantageously, in the formula IIa of said starting material, Ac is (C_1-C_9)acyl, R is a OAc and B' represents a radical of structure (c), in which X_3 is hydrogen or fluorine or methyl and Y is oxygen or, in the formula IIa of said starting material, Ac represents a (C_1-C_9)acyl, R is hydrogen and B' represents a radical of structure (c), in which X_3 is methyl and Y is oxygen.

Particularly advantageous starting materials according to this advantageous embodiment are those of formula IIa wherein:

- R is hydrogen or, preferably, acetyloxy, Ac is acetyl and B' is a radical having the structure (b) wherein X_1 is hydrogen, phenylacetyl or acetyl and Y is an oxygen atom; or
- R is acetyloxy, Ac is acetyl and B' represents a radical having the structure (c), wherein X_3 represents hydrogen or fluorine and Y represents oxygen; or
- R is hydrogen, Ac is acetyl and B' represents a radical having the structure (c), wherein X_3 represents methyl and Y represents oxygen; or
- R is hydrogen or, preferably, acetyloxy, Ac is acetyl and B represents a radical of structure (d), wherein X_1 represents hydrogen, acetyl or phenylacetyl and Y represents oxygen.

Preferred starting materials according to this advantageous embodiment are N^2-acetyl-2',3',5'-tri-O-acetylguanosine, 3',5'-di-O-acetyl-2'-deoxy-5-methyluridine (3',5'-di-O-acetyl-thymidine), 2',3',5'-tri-O-acetyl-5-fluorouridine, 2',3',5'-tri-O-acetyluridine, the 2',3',5'-tri-O-acetylcytidine, the N^4-acetyl-2',3',5'-tri-O-acetylcytidine.

Thus, according to this embodiment, the present invention provides a process for the preparation of a nucleoside of formula Ia



(Ia)

wherein B' represents a radical selected from the group consisting of those having the formulas (b), (c) and (d) as defined above, R represents a hydrogen atom or a group OAc , Ac being a (C_1-C_{18})acyl radical, R_1 and R_2 represent hydrogen or an Ac radical, Ac being as defined above, one of R_1 and R_2 being hydrogen, which comprises treating the corresponding 3',5'-diprotected nucleoside of formula IIa, wherein B', R and Ac are as defined above, with a lipase immobilized on a hydrophobic support in an aqueous solvent at a pH of from 5 to 9.

The selective hydrolysis is carried out as illustrated above, advantageously at a pH of from 6.5 to 7.5, preferably at a pH of about 7 using a lipase from *Candida rugosa* as preferred micro-organism of the genus *Candida*, a lipase from *Pseudomonas fluorescens* or *Pseudomonas cepacia* as preferred micro-organism of the genus *Pseudomonas* or a lipase from porcine pancreas as preferred animal lipase, the aqueous solvent being preferably a phosphate buffer/acetonitrile mixture in the above illustrated ratios and said lipase being immobilized on a gel octyl-agarose or on a Sepabeads resin having a hydrophobicity degree equal to or higher than that of said octyl-agarose gel.

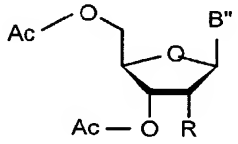
According to a preferred aspect of this embodiment,

- the 2',3',5'-tri-O-acetyl-5-fluorouridine starting material [formula IIa in which Ac is acetyl, R is acetoxy and B' is a radical having the structure (c) in which X_3 is fluorine and Y is oxygen], is treated with a lipase selected from the group consisting of those from *Candida rugosa*, *Pseudomonas fluorescens* and *Pseudomonas cepacia*, whereby a selective 5'-deacylation occurs, said lipase being immobilized on a hydrophobic support;
- the 2',3',5'-tri-O-acetyluridine starting material [formula IIa in which Ac is acetyl, R is acetoxy and B' is a radical having the structure (c) in which X_3 is hydrogen and Y is oxygen], is treated with a lipase from *Candida rugosa*, whereby the selective 5'-deacylation occurs, said lipase being immobilized on a hydrophobic support;
- the 2',3',5'-tri-O-acetyluridine starting material [formula IIa in which Ac is acetyl, R is acetoxy and B' is a radical having the structure (c) in which X_3 is hydrogen and Y is oxygen], is treated with a lipase from *Pseudomonas fluorescens*, whereby a

selective 3'-deacylation occurs, said lipase being immobilized on a hydrophobic support;

- the 3',5'-di-O-acetyl-thymidine [formula IIa in which Ac is acetyl, R is hydrogen and B' is a radical having the structure (c) in which X_3 is methyl and Y is oxygen], is treated with a lipase from porcine pancreas, whereby the selective 3'-deacylation occurs, said lipase being immobilized on a hydrophobic support.

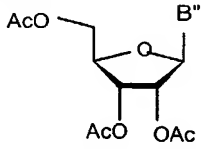
Other interesting starting materials of the process of the present invention are nucleosides of formula IIb



(IIb)

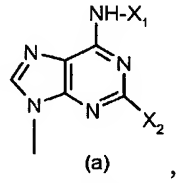
wherein Ac is a (C_1-C_{18})acyl radical, R represents a hydrogen atom or a group OAc, Ac being as defined above, B" represents the radical of a nucleoside base having the structure (a) as defined above.

According to another preferred embodiment, the present invention provides a process for the mono- or di-deacylation in the positions 5' and 3' of a 2',3',5'-tri-O-acylribonucleoside of formula IIb'



(IIb')

wherein Ac is a (C_1-C_{18})acyl radical and B" represents the radical of a nucleoside base having the structure (a)



(a) ,

wherein X_1 represents hydrogen, a (C_1-C_4)alkyl group, an aralkyl group or a group Ac_1, Ac_1 being a (C_1-C_9)acyl group and X_2 represents hydrogen or a (C_1-C_4)alkyl group, which comprises treating said compound of formula IIb' with a lipase immobilized on a hydrophobic support, in an aqueous solvent at a pH of from 5 to 9.

The enzymatic hydrolysis is carried out with a lipase isolable from an animal or microbial source, advantageously from porcine pancreas or from *Pseudomonas fluorescens, Pseudomonas cepacia, Candida rugosa* or *Rhizomucor miehei*. The

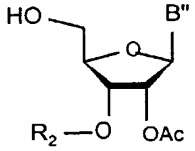
obtention of a 5'-deacylation or of a 3',5'-di-deacylation mainly depends on the enzymatic charge and on the incubation time.

The preferred conditions are as illustrated above, i.e. incubation of the starting nucleoside of formula IIb' in an aqueous solvent, as defined hereinabove, advantageously at a pH of from 6.5 to 7.5, preferably of about 7, at a temperature of from 4 to 40°C, normally at room temperature, for a period of time of from 3 to 80 hours in the presence of the immobilized enzyme or percolation of the solution of the starting nucleoside IIb' through a column containing the immobilized enzyme.

Particularly advantageous starting materials according to this other embodiment are those of formula IIb' wherein B" represents a radical having the structure (a), in which Ac is a (C_1-C_9)acyl, X_1 is hydrogen or Ac_1 as defined above and X_2 is hydrogen.

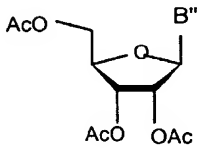
Preferably, according to this other advantageous embodiment, the N-*n*-butanoyl-2',3',5'-tri-O-*n*-butanoyladenosine [formula IIb' in which Ac is *n*-butanoyl, and B" is a radical having the structure (a) in which X_1 is *n*-butanoyl and X_2 is hydrogen] is used as starting material. Said starting material is treated with a lipase selected from the group consisting of those obtainable from porcine pancreas, *Pseudomonas fluorescens, Pseudomonas cepacia, Candida rugosa* or *Rhizomucor miehei*, said lipase being immobilized on a hydrophobic support.

In particular, according to this other advantageous embodiment, the invention provides a process for the preparation of a nucleoside of formula Ib



(Ib)

wherein B" and Ac are as defined above, which comprises treating a compound of formula IIb'



(IIb')

either with a lipase isolable from a micro-organism of the genus P*seudomonas*, or with a lipase of animal or microbial origin immobilized on a hydrophobic support.

Advantageously, a compound of formula IIb' in which Ac is *n*-butanoyl, and B" is a radical having the structure (a) in which X_1 is *n*-butanoyl and X_2 is hydrogen, is treated with a lipase immobilized on a hydrophobic support, in particular from

Candida rugosa or from *Pseudomonas fluorescens*, to obtain a corresponding compound of formula Ib, in which R_2 is *n*-butanoyl.

The product thus obtained may be further submitted to a supplemental hydrolysis with a lipase immobilized on a hydrophobic support, advantageously with a lipase from porcine pancreas, *Pseudomonas fluorescens, Pseudomonas cepacia, Candida rugosa* or *Rhizomucor miehei*, whereby a compound of formula Ib, wherein R_2 is hydrogen is obtained.

When the compound of formula IIb', in which Ac is *n*-butanoyl, and B" is a radical having the structure (a) in which X_1 is *n*-butanoyl and X_2 is hydrogen, is treated with a lipase from *Pseudomonas fluorescens* or, preferably, from *Candida rugosa* immobilized on a hydrophobic support, a corresponding compound of formula Ib, in which R_2 is hydrogen is obtained. To this purpose, is better to use of a lipase from *Candida rugosa* because, after the hydrolysis of the 5'-acyloxy group, that from *Pseudomonas fluorescens* has a period of stasis before hydrolyzing the 3'-acyloxy group.

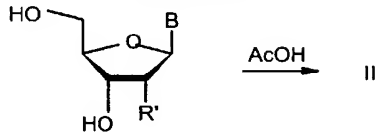
Thus, according to this other advantageous embodiment, the selective hydrolysis of N-*n*-butanoyl-2',3',5'-tri-O-*n*-butanoyladenosine in the presence of a lipase from *Pseudomonas fluorescens* preferably gives the N-*n*-butanoyl-2',3'-di-O-*n*-butanoyladenosine, while the selective hydrolysis in the presence of lipase from *Candida rugosa* preferably affords the N-*n*-butanoyl-2'-O-*n*-butanoyladenosine

Even though it has surprisingly found that any lipase from animal or microbial origin immobilized on a hydrophobic support, in particular lipases from porcine pancreas, *Pseudomonas fluorescens, Pseudomonas cepacia, Candida rugosa* or *Rhizomucor miehei* may generate N-*n*-butanoyl-2'-O-*n*-butanoyladenosine starting from N-*n*-butanoyl-2',3',5'-tri-O-*n*-butanoyladenosine, the most advantageous conditions for this purpose use a lipase from *Candida rugosa* immobilized on a hydrophobic support, preferably on octyl-agarose gel in phosphate buffer at a pH of from 5 to 9, advantageously of from 6.5 to 7.5 with an enzyme charge and incubation time sufficient to assure the 3',5'-hydrolysis. In practice, to a 10-30 (for example 15) mM solution of N-*n*-butanoyl-2',3',5'-tri-O-*n*-butanoyladenosine, prepared by dissolving the calculated amount (for example 246 mg) of N-*n*-butanoyl-2',3',5'-tri-O-*n*-butanoyladenosine, synthesized e.g. as described in PREPARATION VIa below, in 20-50 (for example 30) ml of 25 mM phosphate buffer at a pH of from 5 to 9 (for example 7) containing 30% of CH_3CN, a charge of 20-200 units of expressed lipase from *Candida rugosa* immobilized on gel octyl agarose (for example 2 g of

the enzyme obtained as described in PREPARATION I below) are added. The solution is kept under mechanical stirring at room temperature and the pH is kept constant by automatic titration (addition of a solution of 100 mM NaOH). The progress of the reaction is monitored by HPLC (RP select B column; eluent: 30% CH_3CN – 70% phosphate buffer 10 mM at pH 3.5; flux: 1,2 ml/minute; λ = 260 nm; oven temperature: 30°C). After an incubation of 5-15 hours, the N-*n*-butanoyl-2'-O-*n*-butanoyladenosine is obtained. The N-*n*-butanoyl-2'-O-*n*-butanoyladenosine thus obtained is purified by chromatographic separation by using an eluting mixture in gradient from CH_2Cl_2 100 to CH_2Cl_2-MeOH 90-10.

The products of formula II are known in the literature or can be easily prepared according to known methods, for example by treatment of the corresponding ribonucleoside or 2'-deoxyribonucleoside with a functional derivative of the acid AcOH, Ac being as defined above, optionally in the presence of a tertiary organic base, according to Scheme 1

Scheme 1



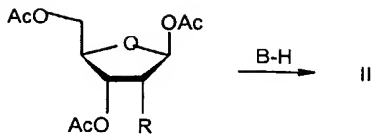
wherein Ac and B are as defined above and R' is H or OH.

Advantageous functional derivatives are the anhydride and the halides, preferably the chloride, even though mixed anhydrides and active esters may be successfully used.

As tertiary organic bases, for example pyridine, dimethylaminopyridine, methylmorpholine, triethylamine and the like may be used. Acylation may be carried out for example according to the general method described by A. Matsuda et al. in Chem. Pharm. Bull., 1988, 36, 945-953. In particular, butylations may be performed as described by J. Wang et al. in Journal Organic Chemistry, 1998, 63, 4850-4853.

Alternatively, advantageously in case of the preparation of compounds of formula II wherein Ac is formyl or, preferably, acetyl, said compounds can be obtained by reaction of tetra-acylribose or triacyl-2-deoxyribose with the selected base according to Scheme 2 as described by G. Gosselin et al. in Journal Medicinal Chemistry, 1987, 30, 982-991.

Scheme 2



II

wherein R and B are as defined above and Ac preferably represents formyl or acetyl. The corresponding starting materials of Scheme 1 are known in the literature or can be easily prepared by known methods. Analogously, the starting compounds of Scheme 2 are known in the literature or can be easily prepared by known methods.

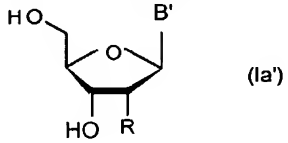
The process of the present invention allows the selective preparation on industrial scale of ribonucleoside derivatives of formula Ia wherein only one of the sugar hydroxy groups is free while the others are O-acylated or, in the case of 2'-deoxynucleoside derivatives, only one of the sugar hydroxy groups is free while the other is O-acylated.

The compounds of formula Ia thus obtained are useful intermediates in the preparation of ribonucleoside or 2'-deoxyribonucleoside derivatives by conversion of the free hydroxy group into a corresponding leaving group which allow the replacement of the hydroxy group for example by hydrogen or by another functional group.

The compounds of formula Ia obtained by hydrolysis catalyzed by a lipase which is selective for one of the groups 3' or 5' may be submitted to a subsequent hydrolysis catalyzed by the other lipase in order to remove also the other acyl group in the position 5' or 3', respectively.

This method may be applied to anyone of the products of formula I, but it is particularly useful when R represent a (C_1-C_{18})acyloxy radical because it allows the preparation of ribonucleoside derivatives in which the hydroxy groups in the 3' an 5' are free while that in position 2' is O-acylated.

Thus, according to another of its aspects, the present invention provides the use of compounds of formula Ia for the preparation of compounds of formula Ia'



(Ia')

wherein R and B' are as defined above, by enzymatic hydrolysis.

According to an advantageous embodiment, compounds of formula Ia, wherein B is as defined above, one of R_1 and R_2 represents hydrogen and the other represents a group Ac as defined above and R represents a group AcO, Ac being as defined above, are used as starting materials for the enzymatic hydrolysis.

The compounds N-*n*-butanoyl-2',3'-di-O-*n*-butanoyladenosine and N-*n*-butanoyl-2'-O-*n*-butanoyladenosine are novel and represent a further object of the present invention.

These compounds are useful intermediates in the synthesis of sodium bucladesine, i.e. of the sodium salt of the cyclic phosphate of N-*n*-butanoyl-2'-O-*n*-butanoyladenosine which can easily prepared from said N-*n*-butanoyl-2'-O-*n*-butanoyladenosine according to known methods.

In the above illustrated process of the present invention, according to the preferred embodiment, said lipase immobilized on a hydrophobic support is selected from the group consisting of lipases from *Candida rugosa*, lipases from *Pseudomonas fluorescens* and lipase from porcine pancreas, said aqueous solvent is phosphate buffer/acetonitrile in a ratio of from 70/30 to 90/10 at a pH of about 7 and said hydrophobic support is selected from the group consisting of octyl-agarose gel and Sepabead resins having a hydrophobicity equal or higher than that of said octyl-agarose gel.

The following examples illustrate the invention without, however, limiting it.

PREPARATION I

Immobilization of the lipase from Candida rugosa

A solution of 139 mg of lipase from *Candida rugosa* (EC 3.1.1.3 Sigma containing 303 mg of protein/g of powder, equal to 0.25U/mg of powder) in 50 ml of phosphate buffer 25 mM at pH 7 is let under stirring for about 30 minutes at room temperature, then 1 g of gel octyl agarose (Octyl Sepharose® CL-4B, Pharmacia Biotech) previously washed first with ethanol, water and, then, with the immobilizing buffer (phosphate buffer 25 mM at pH 7), is added thereto. The mixture is let under stirring for about three hours at room temperature then filtered. The derivative thus obtained, consisting of the resin whereon the enzyme is immobilized, is washed with the minimal amount of water (5 ml). Thus, the lipase from *Candida rugosa* immobilized on Octyl Sepharose® CL-4B, containing 42 mg of protein per gram of gel is obtained in an immobilization yield, calculated as amount of immobilized enzyme, equal to 80% (Bradford assay) and, calculated as expressed activity, equal to about 86%.

PREPARATION II

Immobilization of the lipase from Pseudomonas fluorescens

A solution of 353 mg of lipase from *Pseudomonas fluorescens* (EC 3.1.1.3 Amano containing 120 mg of protein/g of powder, equal to 0.12 U/mg powder) in 50 ml of 25 mM phosphate buffer at pH 7 is let to stand under stirring for about 30 minutes at

room temperature, then 1 g of gel octyl agarose (Octyl Sepharose® CL-4B, Pharmacia Biotech), previously washed first with ethanol, water and then with the immobilizing buffer (25 mM phosphate buffer at pH 7), is added thereto. By operating as described in PREPARATION I, the lipase from *Pseudomonas fluorescens* immobilized on Octyl Sepharose® CL-4B, containing 42 mg of protein per gram of gel is obtained in an immobilization yield, calculated as the amount of immobilized enzyme, equal to about 99% (Bradford assay) and, calculated as expressed activity, equal to about 56%.

PREPARATION III

Immobilization of the lipase from porcine pancreas

A solution of 407 of lipase from porcine pancreas (EC 3.1.1.3 Sigma containing 103 mg of protein/g of powder, equal to 0,24 U/mg powder) in 50 ml of phosphate 25 mM buffer at pH 7 is let to stand under stirring for about 30 minutes at room temperature, then 1 g of gel octyl agarose (Octyl Sepharose® CL-4B, Pharmacia Biotech) previously washed first with ethanol, water, then with the immobilizing buffer (25 mM phosphate buffer at pH 7) is added thereto. Then, by operating as described in PREPARATION I the lipase from porcine pancreas immobilized on Octyl Sepharose® CL-4B, containing 42 mg of protein per gram of gel is obtained in an immobilization yield, calculated as amount of immobilized enzyme, equal to about 15% (Bradford assay) and, calculated as expressed activity, equal to about 70%.

PREPARATION IV

Immobilization of the lipase from Pseudomonas cepacia

A solution of 407 of lipase from *Pseudomonas cepacia* (EC 3.1.1.3 Amano containing 7,6 mg of protein/g of powder, equal to 0,23 U/mg powder) in 50 ml of phosphate 25 mM buffer at pH 7 is let to stand under stirring for about 30 minutes at room temperature, then 1 g of gel octyl agarose (Octyl Sepharose® CL-4B, Pharmacia Biotech) previously washed first with ethanol, water, then with the immobilizing buffer (25 mM phosphate buffer at pH 7) is added thereto. Then, by operating as described in PREPARATION I the lipase from *Pseudomonas cepacia* immobilized on Octyl Sepharose® CL-4B, containing 42 mg of protein per gram of gel is obtained in an immobilization yield, calculated as amount of immobilized enzyme, equal to about 65% (Bradford assay) and, calculated as expressed activity, equal to about 69%.

PREPARATION V

Immobilization of lipase from Rhizomucor miehi

A solution of 2 ml of lipase from *Rhizomucor miehei* (EC 3.1.1.3 Novo Nordisk containing 21 mg of protein/ml of solution, equal to 298 U/ml of solution) in 50 ml of 25 mM phosphate buffer at pH 7 is let to stand under stirring for about 30 minutes at room temperature, then 1 g of resin (Sepabeads FP-RPOD, Resindion - Mitsubishi Chemical Corporation), previously washed first with ethanol, water, then with the immobilizing buffer (25 mM phosphate buffer at pH 7), is added thereto. Then, by operating as described in PREPARATION I, the lipase from *Rhizomucor miehei* immobilized on Sepabeads FP-RPOD, containing 42 mg of protein per gram of resin is obtained in an immobilization yield, calculated as amount of immobilized enzyme, equal to about 39% (Bradford assay) and, calculated as expressed activity, equal to about 450%, value due to the hyperactivation of the enzyme.

By using the same Rhizomucor miehei lipase, an assay has been made in order to compare the specific activity of the enzyme immobilized on hydrophobic supports (gel octyl-agarose, Butyl-Sepabeads, Decaoctyl-Sepabeads) with that of the enzyme in free form (free enzyme) or immobilized on a non hydrophobic resin (Amberlite, Eupergit C). The results of this assay, which show the higher enzymatic activity of the lipase immobilized on a hydrophobic support in respect of those of the same enzyme, free or immobilized on a non-hydrophobic support, are given in Table I and demonstrate that the hydrophobic immobilization support positively influences the enzymatic properties.

Table 1

SUPPORT	IMMOBIL-IZATION YIELD *	CHARGE mg protein/ g derivative	ACTIVITY U/g or U/ml	SPECIFIC ACTIVITY U/mg protein
Free enzyme	-	-	298	14
Octyl-agarose	41%	17.3	917	75.7
Butyl-Sepabeads	29%	12.2	292	24.0
Decaoctyl-Sapebeads	39%	16.3	1010	62.0
Amberlite	98%	41.4	84	2.0
Eupergit C	96%	35.9	16	0.4

* Conditions of immobilization: 42 mg of protein per gram of support; 25°C

PREPARATION VI

N-n-Butanoyl-2',3',5'-tri-O-n-butanoyladenosine

(a) Via acid chloride

To a solution of adenosine (1 g, 3.7 mmoles) in anhydrous pyridine (40 ml) *n*-butanoyl chloride (1.32 ml, 12.8 mmoles) is added at 0°C. The temperature of the mixture is then let to increase to room temperature slowly and kept under stirring for 2 hours. After removal of pyridine under reduced pressure, the crude product is purified by chromatographic technique (J. Wang et al. Journal Organic Chemistry, 1998, 63, 4850-4853). Thus, 850 mg of N-*n*-butanoyl-2',3',5'-tri-O-*n*-butanoyladenosine are obtained

(b) Via anhydride

To a solution of adenosine (6 g, 22 mmoles) in anhydrous pyridine (150 ml) butyric anhydride (73.5 ml, 449 mmoles) is added and the solution is kept under stirring at 118°C for 15 hours. The temperature of the mixture is then let to decrease to room temperature. After removal of the pyridine under reduced pressure, the crude product is resuspended into ethyl acetate and washed with an acidic water solution and then with a sodium bicarbonate solution. After removal of the ethyl acetate from organic phase under reduced pressure, the product is purified both by a chromatographic technique and by a crystallization with *n*-hexane. Thus, 3.1 g of N-*n*-butanoyl-2',3',5'-tri-O-*n*-butanoyladenosine are obtained.

PREPARATION VII

2',3',5'-Tri-O-acetyluridine

To a solution of uridine (2.5 g) and of 4-dimethylaminopyridine (25 mg) in acetonitrile (20 ml), acetic anhydride (3.8 ml) and triethylamine (5.7 ml) are added. After a 30-minute stirring at room temperature, the reaction is complete. After addition of methanol (2 ml), the mixture is kept under stirring for 10 minutes, then the solvent is evaporated by a rotating evaporator to obtain a pale yellow oil consisting of crude 2',3',5'-tris-O-acetyluridine. The crude product is washed with water and methylene chloride, the organic phase is concentrated to dryness and the residue is crystallized from ethanol. Thus, pure 2',3',5'-tris-O-acetyluridine, identical with an authentic sample is obtained.

By operating as described above, starting from 5-fluorouridine, 2'-deoxyuridine, 5-metil-2'-deoxyuridine (thymidine), cytidine, adenosine and, respectively, guanosine, by treatment with the suitable equivalent amounts of acetic anhydride, the 2',3',5'-tri-O-acetyl-5-fluorouridine, 3',5'-di-O-acetyl-2'-deoxyuridine, 3',5'-di-O-acetyl-2'-deoxy-5-methyluridine (3',5'-di-O-acetylthymidine), N^4-acetyl-2',3',5'-tri-O-

acetylcytidine, N^6-acetyl-2',3',5'-tri-O-acetyladenosine and, respectively, N^2-acetyl-2',3',5'-tri-O-acetilguanosine are obtained.

EXAMPLE 1

2',3'-Di-O-acetyluridine

In 40 ml of 25 mM phosphate buffer at pH 7 containing 30% of CH_3CN, 370 mg of 2',3',5'-tri-O-acetyluridine, prepared as described in preparation VI, are dissolved to obtain a 25 mM concentration. To this solution 2 g of lipase from *Candida rugosa* immobilized on gel octyl agarose as described in PREPARATION I are added. The solution is kept under mechanical stirring at room temperature and the pH is kept constant by automatic titration (addition of a solution of 100 mM NaOH). The progress of the reaction is monitored by HPLC (Kromasil C_{18} column; eluent: 20% CH_3CN – 80% phosphate buffer 10 mM at pH 4.2; flux: 1ml/minute; λ = 260 nm). After a 36-hour incubation, the 2',3'-di-O-acetyluridine is obtained in a 86% yield. The 2',3'-di-O-acetyluridine thus obtained is isolated by chromatographic separation from the starting product by using an eluting mixture in gradient from CH_2Cl_2 100 to CH_2Cl_2-MeOH 90-10. The pure product thus obtained is characterized by NMR (OH at δ 5.42) and attribution of the deacetylated position is confirmed by Cosy bidimensional NMR.

^{1}H-RMN (400 MHz $CDCl_3$), δ ppm: 7.71 (d, J=8.2 Hz, 1 H); 6.04 (d, J=5.0 Hz, 1 H); 5.77 (d, J=8.2 Hz, 1 H); 5.42 (s, 1 H); 5.48-5.46 (m, 2 H); 4.21 (m, 1 H); 3.95-3.86 (dd system ABX, J=1.7, 12.0 Hz, 2 H); 2.13 (s, 3 H); 2.08 (s, 3 H).

EXAMPLE 2

2',5'-di-O-acetyluridine

By operating as described in EXAMPLE 1, to the 25 mM solution of 2',3',5'-tri-O-acetyluridine prepared as described in PREPARATION VI, 2 g of lipase from *Pseudomonas fluorescens* immobilized on gel octyl agarose as described in PREPARATION II are added, then the mixture is treated as in EXAMPLE 1. After a 64-hour incubation, the 2',5'-di-O-acetyluridine is obtained in a 85% yield. The 2',5'-di-O-acetyluridine thus obtained is isolated by chromatographic separation from the starting product by using an eluting mixture in gradient from CH_2Cl_2 100 to CH_2Cl_2-MeOH 90-10. The pure product thus obtained is characterized by NMR (OH at δ 5.42) and the attribution of the deacetylated position is confirmed by Cosy bidimensional NMR.

^{1}H-NMR (400 MHz $CDCl_3$), δ ppm: 7.69 (d, J=8.1 Hz, 1 H); 6.03 (d, J=5.3 Hz, 1 H); 5.77 (d, J=8.1 Hz, 1 H); 5.48-5.44 (m, 2 H); 4.21 (m, 1 H); 3.95-3.86 (dd system ABX, J=1.8, 12.0 Hz, 2 H); 3.48 (s, 1 H); 2.13 (s, 3 H); 2.08 (s, 3 H).

EXAMPLE 3

5'-O-acetylthimidine

By operating as described in EXAMPLE 1, to the 25 mM solution of 3',5'-di-O-acetylthymidine obtained as described in PREPARATION VI, prepared by dissolving 163 mg of 3',5'-di-O-acetylthymidine in 20 ml of 25 mM phosphate buffer at pH 7 containing 30% of CH_3CN, 2 g of lipase from porcine pancreas immobilized on gel octyl agarose as described in PREPARATION III are added, then the mixture is treated as in EXAMPLE 1. After a 51-hour incubation, the 5'-O-acetylthymidine is obtained in a 36% yield. The 5'-O-acetylthymidine thus obtained is isolated by chromatographic separation from the starting product by using an eluting mixture in gradient from CH_2Cl_2 100 to CH_2Cl_2-MeOH 90-10. The pure product thus obtained is characterized by NMR and the attribution of the deacetylated position is confirmed by Cosy bidimensional NMR.

EXAMPLES 4-6

2',3'-Di-O-acetyl-5-fluorouridine

By operating as described in EXAMPLE 1, to a 25 mM solution of 2',3',5'-tri-O-acetyl-5-fluorouridine prepared by dissolving 191 mg of 2',3',5'-tri-O-acetyl-5-fluorouridine, obtained as described in PREPARATION VI, in 20 ml of 25 mM phosphate buffer at pH 7 containing 30% of CH_3CN, 2 g of lipase from *Candida rugosa* or *Pseudomonas cepacia* or *Pseudomonas fluorescens* immobilized on gel octyl agarose as described in PREPARATION I, IV and II, respectively, are added, then the mixture is treated as in EXAMPLE 1. After a 48-hour incubation with *Candida rugosa*, the 2',3'-di-O-acetyl-5-fluorouridine is obtained in a 56% yield (EXAMPLE 4). After a 24-hour incubation with *Pseudomonas cepacia* or *Pseudomonas fluorescens*, the 2',3'-di-O-acetyl-5-fluorouridine is obtained in a 49% (EXAMPLE 5) and 60% (EXAMPLE 6) yield respectively The 2',3'-di-O-acetyl-5-fluorouridine thus obtained is isolated by chromatographic separation from the starting product by using an eluting mixture in gradient from CH_2Cl_2 100 to CH_2Cl_2-MeOH 90-10. The pure product thus obtained is characterized by NMR (OH at δ 4.16) and the attribution of the deacetylated position is confirmed by Cosy bidimensional NMR.

^{1}H-NMR (400 MHz $CDCl_3$), δ ppm: 8.03 (s, 1 H); 6.07 (d, J=5.1 Hz, 1 H); 5.32-5.35 (m, 2 H); 3.71-3.82 (dd system ABX, J=1.7, 11.0 Hz, 2 H); 3.55 (m, 1 H); 2.13 (s, 3 H); 2.03 (s, 3 H).

EXAMPLE 7

N-n-butanoyl-2',3'-di-O-n-butanoyladenosine

By operating as described in EXAMPLE 1, to the 15 mM solution of N-*n*-butanoyl-2',3',5'-tri-O-*n*-butanoyladenosine, obtained as described in PREPARATION VI, prepared by dissolving 246 mg of N-*n*-butanoyl-2',3',5'-tri-O-*n*-butanoyladenosine in 30 ml of 25 mM phosphate buffer at pH 7 containing 30% of CH_3CN, 1 g of lipase from *Pseudomonas fluorescens* immobilized on gel octyl agarose as described in PREPARATION II are added, then the mixture is treated as in EXAMPLE 1. The progress of the reaction is monitored by HPLC (RP select B column; eluent: 30% CH_3CN – 70% phosphate buffer 10 mM at pH 3.5; flux: 1,2 ml/minute; λ = 260 nm; oven temperature: 30°C). After a 8-hour incubation, the N-*n*-butanoyl-2',3'-di-O-*n*-butanoyladenosine is obtained in a 78% yield. The N-*n*-butanoyl-2',3'-di-O-*n*-butanoyladenosine thus obtained is isolated by chromatographic separation from the starting product by using an eluting mixture in gradient from CH_2Cl_2 100 to CH_2Cl_2-MeOH 90-10. The pure product thus obtained is characterized by NMR (OH at δ 5.76 ppm) and the attribution of the deacetylated position is confirmed by Cosy bidimensional NMR.

^{1}H-NMR (400 MHz $CDCl_3$), δ ppm: 8.69 (s, 1 H); 8.04 (s, 1 H);6.06 (d, 1 H); 6.03 (t, 1 H); 5.72 (m, 1 H); 4.37 (m, 1 H); 4.02-3.98 (dd system ABX, 2 H); 3.55 (m, 1 H); 2.92 (t, 2 H); 2.40 (t, 2 H); 2.24 (t, 2 H); 1.79 (m, 2 H); 1.72 (m, 2 H); 1.57 (m, 2 H); 1.05 (t, 3 H); 1.01 (t, 3 H); 0.89 (t, 3 H)

CLAIMS

1. A process for the selective deacylation in position 5' or 3', or in both of them, of a 2',3',5'-tri-O-acylribonucleoside or in position 5' or 3' of a 3',5'-di-O-acyl-2'-deoxyribonucleoside, which comprises treating said 2',3',5'-tri-O-acylribonucleoside or 3',5'-di-O-acyl-2'-deoxyribonucleoside with a lipase immobilized on a hydrophobic support in an aqueous solvent at a pH of from 5 to 9.

2. A process according to claim 1, wherein said lipase is selected from the group consisting of Candida Rugosa Lipase, Pseudomonas Fluorescens Lipase and Porcine Pancreas Lipase.

3. A process according to anyone of claims 1 and 2, wherein the aqueous solvent consists of 70-80% water and 30-20% of a water-miscible organic solvent.

4. A process according to claim 3, wherein said water-miscible solvent is a polar aprotic solvent.

5. A process according to claim 4, wherein said polar aprotic solvent is acetonitrile.

6. A process according to anyone of claims 1 to 5, wherein the pH of said aqueous solvent is of from 6.5 to 7.5.

7. A process according to anyone of claims 1 to 6, wherein the reaction is performed at a temperature of from 4 to 50°C.

8. A process according to anyone of claims 1 to 7, wherein said hydrophobic support is an octyl-agarose gel or a Sepabeads resin having a hydrophobicity degree equal or higher to that of said octyl-agarose gel.

9. A process according to anyone of claims 1 to 8, wherein said lipase is of microbial or animal origin.

10. A process according to claim 9, wherein said lipase of microbial origin is obtainable from a micro-organism of the genus selected from the group consisting of *Candida, Pseudomonas, Rhizomucor, Humicola, Aspergillus, Rhizopus, Mucor.*

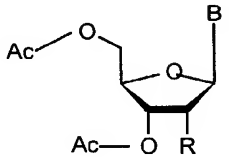
11. A process according to claim 10, wherein said micro-organism of the genus *Candida* is *Candida rugosa.*

12. A process according to claim 10, wherein said micro-organism of the genus *Pseudomonas* is *Pseudomonas fluorescens* or *Pseudomonas cepacia.*

13. A process according to claim 10, wherein said micro-organism of the genus *Rhizomucor* is *Rhizomucor miehei.*

14. A process according to claim 9, wherein said lipase of animal origin is obtainable from porcine pancreas.

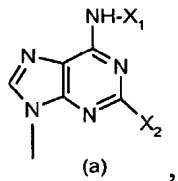
15. A process according to anyone of claims 1 to 14, wherein a compound of formula II



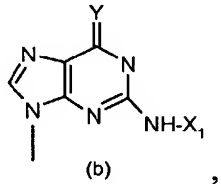
(II)

wherein B represents the radical of a nucleoside base, Ac is a (C_1-C_{18})acyl radical, R represents a hydrogen atom or a group OAc, Ac being as defined above, is used as starting material

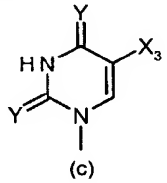
16. A process according to claim 15, wherein in the formula II of said starting material B represents the radical of a nucleoside base selected from the group consisting of the radicals having the structures (a)-(d)



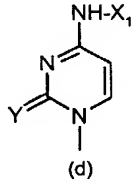
(a) ,



(b) ,



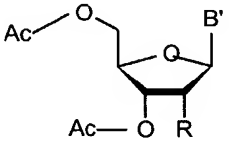
(c) ,



(d) ,

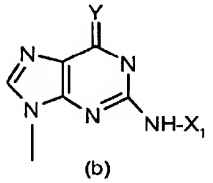
wherein X_1 represents hydrogen, a (C_1-C_4)alkyl group, an aralkyl group or a group Ac_1, Ac_1 being a (C_1-C_9)acyl group, X_2 represents hydrogen or a (C_1-C_4)alkyl group, X_3 represents hydrogen, a (C_1-C_4)alkyl group or a halogen atom and Y represents an oxygen or sulfur atom.

17. A process according to anyone of claims 1 to 16, wherein a nucleoside of formula IIa

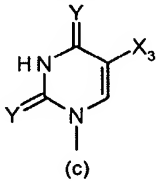


(IIa)

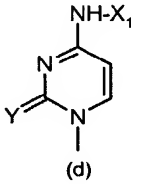
wherein Ac is a (C_1-C_{18})acyl radical, R represents a hydrogen atom or a group OAc, Ac being as defined above, B' represents the radical of a nucleoside base selected from those having the structures (b), (c) and (d)



(b) ,



(c) ,



(d) ,

wherein X_1 represents hydrogen, a (C_1-C_4)alkyl group, an aralkyl group or a group Ac_1,Ac_1 being a (C_1-C_9)acyl group, X_3 represents hydrogen, a (C_1-C_4)alkyl group or a halogen atom and Y represents an oxygen or sulfur atom, is treated with a lipase immobilized on a hydrophobic support in an aqueous solvent at a pH of from 5 to 9, whereby a selective 5' or 3' deacylation occurs.

18. A process according to claim 17, wherein a compound of formula IIa, wherein B' represents a radical selected from the group consisting of those having the above-defined structures (b), (c) and (d), in which X_1 is hydrogen or a (C_1-C_9)acyl, X_3 is hydrogen, fluorine or methyl and Y is oxygen is used as starting material.

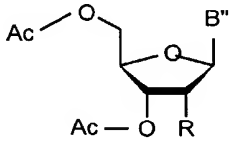
19. A process according to claim 17, wherein a compound of formula IIa, wherein R is hydrogen or acetyloxy, Ac is acetyl and B' is a radical having the structure (b) wherein X_1 is hydrogen, phenylacetyl or acetyl and Y is an oxygen atom is used as starting material.

20. A process according to claim 17, wherein a compound of formula IIa, wherein R is acetyloxy, Ac is acetyl and B' is a radical having the structure (c) wherein X_3 is methyl and Y is oxygen atom is used as starting material.

21. A process according to claim 17, wherein a compound of formula IIa, wherein R is hydrogen or acetyloxy, Ac is acetyl and B' is a radical having the structure (d) wherein X_1 is hydrogen, phenylacetyl or acetyl and Y is oxygen is used as starting material.

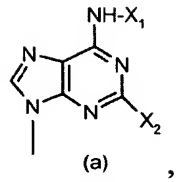
22. A process according to claim 17, wherein a member selected from the group consisting of N^2-acetyl-2',3',5'-tri-O-acetylguanosine, 3',5'-di-O-acetyl-2'-deoxy-5-methyluridine (3',5'-di-O-acetyl-thymidine), 2',3',5'-tri-O-acetyl-5-fluorouridine, 2',3',5'-tri-O-acetyluridine, 2',3',5'-tri-O-acetylcytidine, and N^4-acetyl-2',3',5'-tri-O-acetylcytidine is used as starting material.

23. A process according to anyone of claims 1 to 16, wherein a nucleoside of formula IIb



(IIb)

wherein Ac is a (C_1-C_{18})acyl radical, R represents a hydrogen atom or a group OAc, Ac being as defined above, B" represents the radical of a nucleoside base having the structure (a)



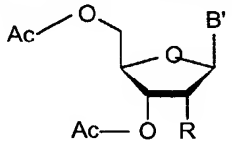
(a) ,

wherein X_1 represents hydrogen, a (C_1-C_4)alkyl group, an aralkyl group or a group Ac_1, Ac_1 being a (C_1-C_9)acyl group and X_2 represents hydrogen or a (C_1-C_4)alkyl group, is treated in an aqueous solvent at a pH of from 5 to 9 with a lipase immobilized on a hydrophobic support.

24. A process according to claim 23 wherein a nucleoside of formula IIb in which Ac is *n*-butanoyl, and B" is a radical having the structure (a), in which X_1 is *n*-butanoyl and X_2 is hydrogen, is treated with a lipase from *Pseudomonas fluorescens*, whereby the selective 5'-deacylation occurs, said lipase being immobilized on a hydrophobic support.

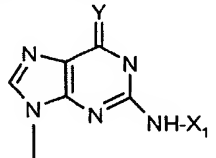
25. A process according to claim 23 wherein a nucleoside of formula IIb in which Ac is *n*-butanoyl, and B" is a radical having the structure (a), in which X_1 is *n*-butanoyl and X_2 is hydrogen, is treated with a lipase from *Candida rugosa*, whereby the selective 3',5'-di-deacylation occurs, said lipase being immobilized on a hydrophobic support.

26. A process according to claim 17, wherein a compound of IIa

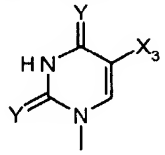


(IIa)

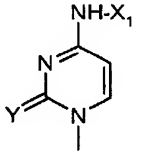
wherein Ac is a (C_1-C_{18})acyl radical, R represents a hydrogen atom or a group OAc, Ac being as defined above, B' represents the radical of a nucleoside base selected from those having the formulas (b), (c) and (d)



(b) ,

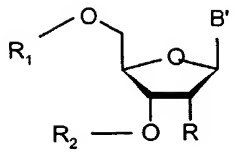


(c) ,



(d) ,

wherein X_1 represents hydrogen, a (C_1-C_4)alkyl group, an aralkyl group or a group Ac_1,Ac_1 being a (C_1-C_9)acyl group, X_3 represents hydrogen, a (C_1-C_4)alkyl group or a halogen atom and Y represents an oxygen or sulfur atom, is treated with a lipase immobilized on a hydrophobic support in an aqueous solvent at a pH of from 5 to 9, whereby a compound of formula Ia



(Ia)

in which B' and R are as defined above, R_1 and R_2 represent hydrogen or Ac, one of R_1 and R_2 being hydrogen, is obtained.

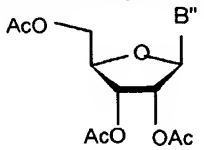
27. A process according to claim 26, wherein a compound of formula IIa in which Ac is acetyl, R is acetoxy and B' is a radical having the structure (c) in which X_3 is fluorine and Y is oxygen, is treated with a lipase selected from the group consisting of those from *Candida rugosa*, *Pseudomonas fluorescens* and *Pseudomonas cepacia*, said lipase being immobilized on a hydrophobic support, whereby a selective 5'-deacylation occurs and a corresponding nucleoside of formula Ia, in which R_1 is hydrogen and R_2 is acetyl is obtained.

28. A process according to claim 26, wherein a compound of formula IIa in which Ac is acetyl, R is acetoxy and B' is a radical having the structure (c) in which X_3 is hydrogen and Y is oxygen, is treated with a lipase from *Candida rugosa*, said lipase being immobilized on a hydrophobic support, whereby a selective 5'-deacylation occurs and a corresponding nucleoside of formula Ia, in which R_1 is hydrogen and R_2 is acetyl is obtained.

29. A process according to claim 26, wherein a compound of formula IIa in which Ac is acetyl, R is acetoxy and B' is a radical having the structure (c) in which X_3 is hydrogen and Y is oxygen, is treated with a lipase from *Pseudomonas fluorescens*, said lipase being immobilized on a hydrophobic support, whereby a selective 3'-deacylation occurs and a corresponding nucleoside of formula Ia, in which R_2 is hydrogen and R_1 is acetyl is obtained.

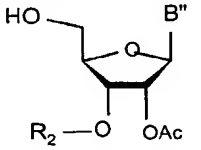
30. A process according to claim 26, wherein a compound of formula IIa in which Ac is acetyl, R is hydrogen and B' is a radical having the structure (c) in which X_3 is methyl and Y is oxygen, is treated with a lipase from porcine pancreas, said lipase being immobilized on a hydrophobic support, whereby a selective 3'-deacylation occurs and a corresponding nucleoside of formula Ia, in which R_2 is hydrogen and R_1 is acetyl is obtained.

31. A process according to claim 23, wherein a compound of formula IIb'



(IIb')

wherein B" and Ac are as defined above, is treated with a lipase immobilized on a hydrophobic support, whereby a nucleoside of formula Ib



(Ib)

wherein R_2 is hydrogen or Ac, is obtained.

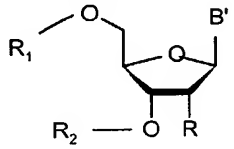
32. A process according to claim 31, wherein a compound of formula IIb' in which Ac is *n*-butanoyl, and B" is a radical having the structure (a) in which X_1 is *n*-butanoyl and X_2 is hydrogen, is treated with a lipase from *Pseudomonas fluorescens* immobilized on a hydrophobic support, whereby a corresponding compound of formula Ib, in which R_2 is *n*-butanoyl is obtained.

33. A process according to claim 31, wherein a compound of formula IIb' in which Ac is *n*-butanoyl, and B" is a radical having the structure (a) in which X_1 is *n*-butanoyl and X_2 is hydrogen, is treated with a lipase from *Candida rugosa* immobilized on a hydrophobic support, whereby a corresponding compound of formula Ib, in which R_2 is hydrogen is obtained.

34. N-*n*-butanoyl-2',3'-di-O-*n*-butanoyladenosine.

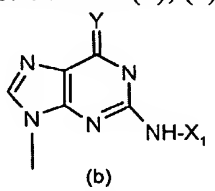
35. N-*n*-butanoyl-2'-O-*n*-butanoyladenosine.

36. Use of the compounds of formula Ia

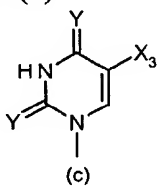


(Ia)

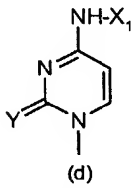
wherein R represents a hydrogen atom or a group OAc, Ac being a (C_1-C_{18})acyl radical as defined above, B' represents the radical of a nucleoside base selected from those having the structures (b), (c) and (d)



(b),

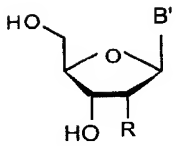


(c),



(d),

wherein X_1 represents hydrogen, a (C_1-C_4)alkyl group, an aralkyl group or a group Ac_1, Ac_1 being a (C_1-C_9)acyl group, X_3 represents hydrogen, a (C_1-C_4)alkyl group or a halogen atom and Y represents an oxygen or sulfur atom, for the preparation of compounds of formula Ia'



(Ia')

wherein R and B' are as defined above, by enzymatic hydrolysis.

37. Use according to claim 36 wherein, in the formula Ia', R represents a group OAc.

38. Use according to claim 37 wherein said group OAc is acetyloxy.

INTERNATIONAL SEARCH REPORT

Inte nal Application No
PCT/IB 03/00056

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P19/38 C12P19/40 C07H19/00 C12N11/00 C07H19/167
C07H19/173

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C07H C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	GARCIA J. ET AL.: "Building blocks for the Solution Phase Synthesis of Oligonucleotides: Regioselective Hydrolysis of 3',5'-Di-O-levulinylnucleosides Using an Enzymatic Approach." J. ORG. CHEM., vol. 67, no. 13, 28 June 2002 (2002-06-28), pages 4513-4519, XP002237341 the whole document --- -/--	1-38

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 April 2003	17/04/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Douschan, K

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IGLESIAS L. E. ET AL.: "Complete and regioselective deacylation of peracetylated uridines using a lipase." BIOTECHNOLOGY LETTERS, vol. 22, 2000, pages 361-365, XP009008773 cited in the application see especially p. 361, 362 and 364 the whole document	1-38
X	SINGH H. K. ET AL.: "Enzymatic regioselective deacylation of 2',3',5'-tri-O-acylribonucleosides: enzymatic synthesis of 2',3'-di-O-acylribonucleosides." TETRAHEDRON LETT., vol. 34, no. 33, 1993, pages 5201-5204, XP009008776 cited in the application the whole document	1-38
X	UEMURA A. ET AL.: "Regioselective deprotection of 3',5'-O-acylated pyrimidine nucleosides by lipase and esterase." TETRAHEDRON LETT., vol. 30, 1989, pages 3819-3820, XP009008775 cited in the application the whole document	1-38
X	US 5 712 099 A (SCHERING AG) 27 January 1998 (1998-01-27) cited in the application the whole document	1-38

INTERNATIONAL SEARCH REPORT

ɔnal Application No
PCT/IB 03/00056

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		AT 169928 T	15-09-1998
		DE 59503274 D1	24-09-1998
		DK 759926 T3	25-05-1999
		WO 9532212 A1	30-11-1995
		EP 0759926 A1	05-03-1997
		ES 2123246 T3	01-01-1999
		JP 10507063 T	14-07-1998